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Epstein-Barr Virus Nuclear Antigen 2 and Latent Membrane Protein Independently Transactivate p53 through Induction of NF-κB Activity

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B-cell immortalization by Epstein-Barr virus (EBV) is dependent on permanent control of the cellular processes which normally regulate cell division and apoptosis, functions possessed by p53 in a number of normal cell types. In studies initiated to evaluate relationships between EBV latent genes and p53, p53 levels were found to increase approximately 10-fold 4 to 5 days after EBV infection of purified resting human B cells; the induced p53 was transcriptionally active. Latent membrane protein 1 and, to a lesser extent, EBV nuclear antigen 2 mediated the increase in p53 levels via activation of the NF-κB transcription factor.

Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, is closely associated with a number of lymphoid and epithelial malignancies, including endemic Burkitt's lymphoma, a number of T-cell lymphomas, several subtypes of Hodgkin's disease, undifferentiated nasopharyngeal carcinoma, and certain other carcinomas (11, 27, 40, 51). Particularly striking is the frequent presence of the EBV genome in the lymphomas that occur in immunocompromised individuals, including AIDS patients and transplant recipients (6, 21, 25, 56).

More than half of all human malignancies, regardless of the cell of origin, exhibit mutations in the p53 tumor suppressor gene. Among EBV-associated tumors, p53 mutations are frequent in Burkitt's lymphoma (12, 14, 61), moderately common in AIDS-associated lymphomas (5, 36, 45) and Hodgkin's disease Reed Sternberg cells (57), and infrequent in nasopharyngeal carcinoma (9, 52) and posttransplant lymphomas (8). Although the functions of p53 and the mechanisms of its actions are not fully understood, numerous studies have shown that wild-type (wt) p53 regulates cell cycle progression, prevents unrestricted cell division, and induces apoptosis in some cell types with damaged DNA (29, 39). Such functions would prevent unregulated or abnormal cell growth and retard the development of tumors.

EBV infection of normal B lymphocytes in vitro generates latently infected immortal B-lymphoblastoid cell lines (LCL); this process requires the virus to permanently control the cellular processes which regulate cell division and apoptosis, functions which are p53 dependent in a number of normal cell types. The present studies were initiated to evaluate the relationships between EBV genes and p53 during the earliest stages of EBV infection and transformation of human B cells.

EBV infection of resting B cells increases cellular levels of transcriptionally active p53. We found low levels of p53 in dense resting (nonactivated) human B cells purified from tonsils (32) and higher levels in both short- and long-term LCL established by in vitro infection with EBV (data not shown), findings which are consistent with other reports (4, 53). Exons 5 to 8 of four such LCL were amplified by PCR and directly sequenced (AmpliTag cycle sequencing kit; Perkin-Elmer Cetus), using primers lying just outside the exon boundaries, and were found to be wt (data not shown), a result that was anticipated from earlier studies (12). These data suggested that EBV infection increases p53 levels. In order to evaluate this possibility and determine the time course of p53 induction, we incubated purified EBV strain B95-8 (37) with purified resting human tonsil B cells and periodically took samples for assessment of p53 expression by the Western blotting technique. For the latter, DO-1 monoclonal antibody (Oncogene Science) was used, and detection was carried out with peroxidase-labeled anti-mouse immunoglobulin G (Kirkegaard and Perry Laboratories, Inc.) by using the ECL system (Amersham.) As is evident from Fig. 1A, EBV infection of resting B cells increased p53 levels beginning at approximately 24 h after infection, a time which antedates EBV-induced cellular DNA synthesis by at least 24 h (references 2 and 42, and data not shown). Peak p53 levels (approximately 10-fold greater than those of resting B cells) occurred 4 to 5 days after EBV infection in a number of experiments of this type. After this time, p53 levels declined slightly and then remained unchanged for at least 5 months, and probably indefinitely, since the levels at this time point were comparable to those in the LCL preparations which have been examined. Uninfected resting B cells incubated for 6 days showed no changes in p53 levels (data not shown), despite the slow progressive death of the cells (approximately 50% cell death by 6 days), confirming that the increase in p53 levels is EBV specific. While these studies were in progress, two other groups reported that p53 levels increase to peak levels several days after primary EBV infection (4, 53), data which are comparable to the present results. One of these studies reported that elevated p53 levels were maintained (4), which is in agreement with our results, while the other found that p53 levels progressively declined and became undetectable in LCL 4 months after infection (53); the reasons for these differences are not apparent.

Several DNA tumor viruses, including simian virus 40, adenovirus type 5, and human papillomavirus types 16 and 18, encode proteins which bind to, dysregulate, and/or inactivate p53 (30, 31, 46, 48, 60, 65). EBV, like these viruses, overcomes normal cellular regulatory mechanisms, some of which are p53 dependent, leading to immortal growth and the prevention of normal cell death. With regard to p53 and EBV, it has also been reported that EBV nuclear antigen LP (EBNA-LP), a latent EBV gene product (54), and the protein encoded by the

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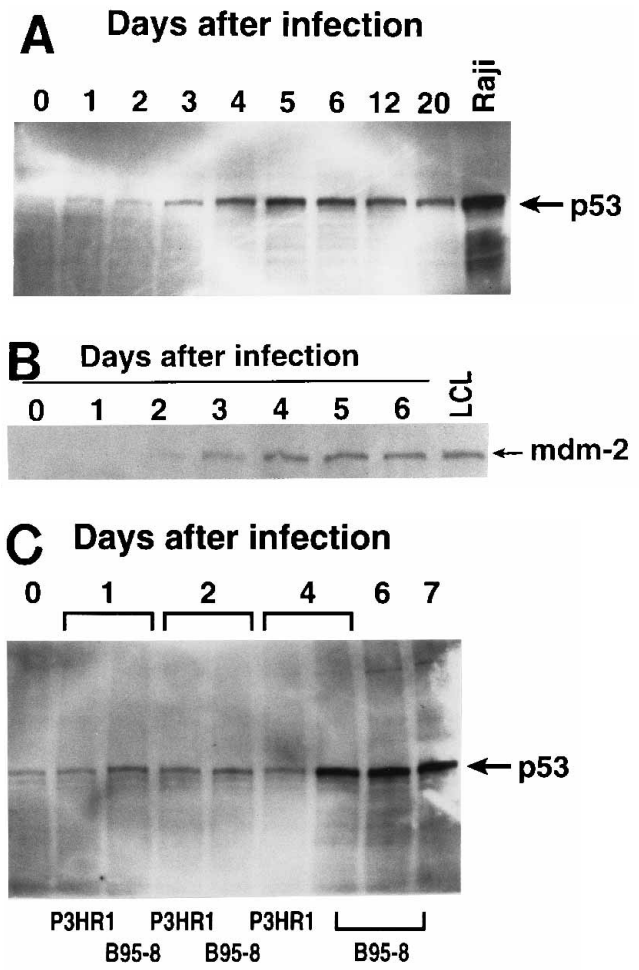

FIG. 1. EBV infection of purified resting human B cells increases cellular levels of transcriptionally active p53 and is dependent on viral gene expression. (A) Total cellular extracts from 5×10^5 cells were prepared at various times after infection with EBV strain B95-8 and assessed for p53 protein by the Western blotting procedure with the DO-1 p53 monoclonal antibody. (B) Western blotting studies of mdm-2 expression at various times after infection, using a monoclonal antibody directed against mdm-2. (C) Western blotting studies of p53 expression at various times after incubation of resting B cells with EBV strain B95-8 or P3HR1.

BZLF-1 gene, a lytic-cycle protein, bind p53 (66), but these studies have not been confirmed and the functional consequences of such interactions have not been examined. In the present studies, we assessed expression of mdm-2 and p21/WAF1, two cellular genes which are transcriptionally activated by wt p53 (10, 63), during the early stages of EBV infection of resting human B cells. As shown in the Western blotting study with mdm-2 monoclonal antibody (Oncogene Science) depicted in Fig. 1B, mdm-2, which was undetectable in purified resting human B cells, increased with the same time course as p53 after EBV infection. The other p53-induced gene, p21/WAF1, behaved similarly; p21/WAF1 mRNA levels, which were very low in resting B cells, as determined by reverse transcription-PCR followed by densitometry, increased 68-fold (adjusted for a modest increase in rpL32 mRNA levels) 5 days after EBV infection (data not shown). These findings indicate that the p53 induced by EBV is transcriptionally active. More importantly, they show that the earliest stages of EBV infection and transformation of resting B cells are not dependent on p53 inactivation by an EBV latent gene product or a cellular protein expressed during infection; similar conclusions have recently been reached by other investigators (4, 53).

The EBV-induced increase in p53 levels in resting B cells is dependent on viral gene expression. Binding of ligands to the CD21 EBV receptor on the cell surface triggers intracellular signaling pathways which modulate B-cell activation (13, 38). In order to determine whether the increased p53 levels observed after EBV infection of resting B cells reflected CD21-initiated signal transduction events or instead were dependent on viral gene expression, purified resting B cells were incubated with EBV strain P3HR1 (37); this EBV strain, although lacking the ability to transform B cells (19) (also verified here), is able to transduce signals after binding to the B-cell surface (15, 24). EBV strain P3HR1 did not increase p53 levels in purified resting B cells, as shown in Fig. 1C. Identical results were obtained with nontransforming UV-irradiated EBV strain B95-8 (data not shown). These studies show that p53 induction is not due to triggering of intracellular signaling pathways by the binding of EBV to CD21 on the cell surface.

EBNA-2 and LMP1 independently mediate the increase in p53 levels after EBV infection of resting B cells. The timing of the observed increases in p53 in resting B cells following EBV infection suggested that EBNA-2 and latent membrane protein 1 (LMP1) might be involved, since EBNA-2 is first detectable 12 to 24 h after infection and peak LMP1 levels are reached 4 to 6 days after infection (1, 2, 42, 50). EBNA-2 is further implicated by the fact that P3HR1 is a deletion mutant lacking the gene coding for EBNA-2 (19). In order to evaluate this possibility, purified resting human B cells were transfected with expression plasmids encoding EBNA-2 (pSG5-EBNA-2A or SV-EBNA-2 [59]) or LMP1 (pSV2gpMTLM [58]), with both of these plasmids together, or with control plasmids pSV2gpt (35) and pSG5 (17) by electroporation in a Bio-Rad Gene Pulser (10 μg of DNA, balanced by addition of vector DNA; 4-mm-gap cuvettes; 320 V and 960 μF). Trivial expression of transfected plasmid DNAs in resting B cells was observed unless the cells were pretreated with recombinant gp105, the ligand-binding portion of gp350/220 (55), in immobilized form on tissue culture dishes (40 ng/ml for 20 h), as recently reported for the complete gp350/220 glycoprotein (50). Under such conditions, the transfection efficiency increased to 2 to 5%, as assessed with a transfected pCMV-β-gal plasmid. Cadmium chloride (6 μM) was added 24 h posttransfection for induction, and total RNA was prepared (approximately 2×10^6 cells per sample; Micro-scale Total RNA Separator Kit, Clontech Laboratories) from live cells by the use of Ficoll 72 h after transfection. Reverse transcription-PCR (10 μg of cDNA per sample) (32) was employed to assess the expression of p53, EBNA-2, LMP1, and rpL32, a housekeeping gene. Upstream and downstream primers and amplified fragment lengths were as follows: 5′-GTTGGCTCTGACTGTACCA-3′ and 5′-AAGGCCTCATTCAGCTCTC-3′ (371 bp) for p53; 5′-CCGCAGGGATGCCTGGACA-3′ and 5′-AGGCCTTGGTGGCATCATG-3′ (348 bp) for EBNA-2; 5′-GGTCTCTGGATCTACTTATT-3′ and 5′-TCCACTCACGAGCAGGAGGT-3′ (330 bp) for LMP1; and 5′-ATGAATTCTCCTTCTCGGCATCATGGC-3′ and 5′-CGGGATCCAGTTACGCTTAATT-3′ (133 bp) for rpL32. PCR products, after electrophoresis on 1% agarose gels, were visualized with ethidium bromide.

As is evident from Fig. 2, EBNA-2 expression in purified resting human B cells increased p53 transcription; densitometric analysis documented a twofold increase. A 5-fold increase occurred in the LMP1-transfected cells, and a 10-fold increase was found in primary B cells transfected with both latent genes.

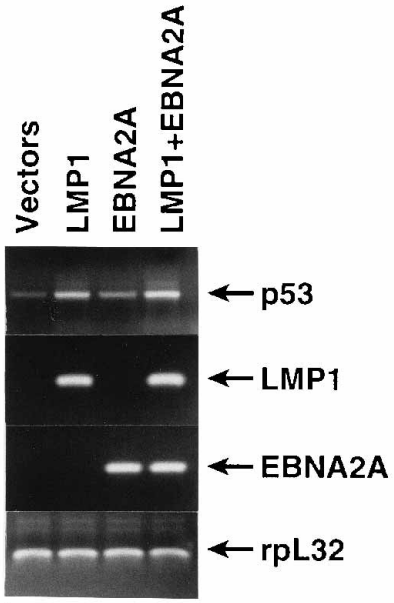

FIG. 2. EBNA-2 and LMP1 independently mediate the increase in p53 levels after EBV infection of resting B cells. Shown are the results of reverse transcription-PCR analysis of p53, EBNA-2, LMP1, and rpL32 expression 72 h after transfection, by electroporation, of gp105-pretreated resting B cells. Vectors, cells transfected with both pSV2gpt and pSG5.

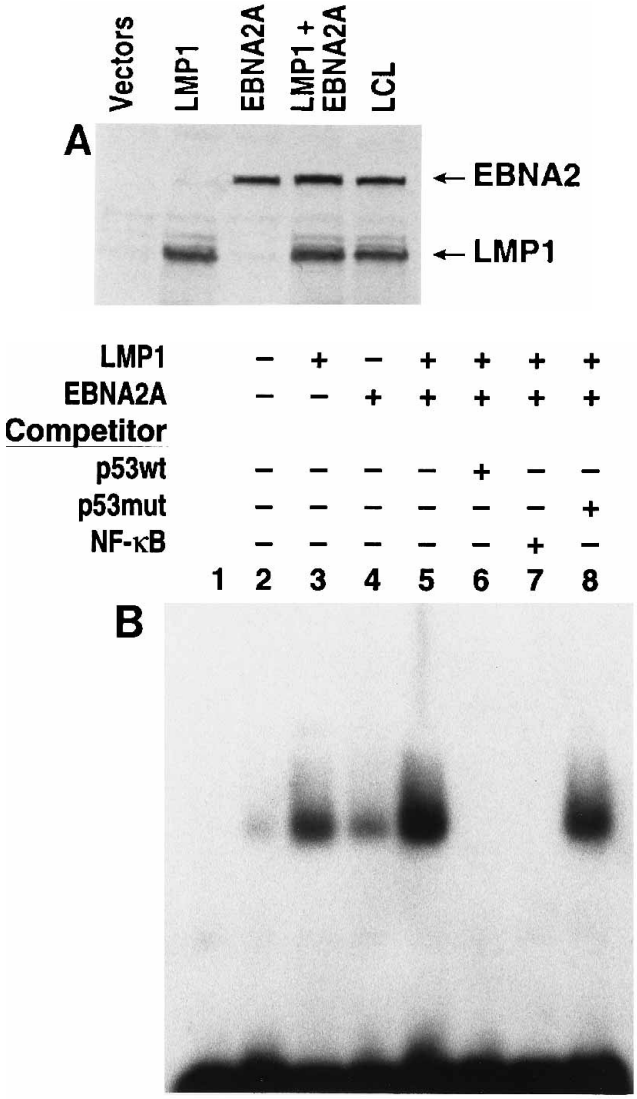

FIG. 3. LMP1 and EBNA-2A expressed in transfected 293 cells trigger the expression of nuclear proteins which bind to the NF-κB site in the p53 promoter. (A) Western blotting analysis of EBNA-2 and LMP1 expression in 293 cells 72 h after transfection, using the PE2 and S12 monoclonal antibodies to EBNA-2 and LMP1, respectively. Vectors, cells transfected with both pSV2gpt and pSG5. (B) Gel shift analysis of nuclear proteins binding to the wt NF-κB sequence in the p53 promoter 72 h after transfection of 293 cells with EBNA-2, LMP1, or both EBNA-2 and LMP1. Competition studies were carried out with 50-fold excesses of the unlabeled wt NF-κB p53 probe, a mutant (mut) p53 probe, and a consensus probe (NF-κB).

These are previously unreported actions of these EBV latent genes.

EBNA-2 and LMP1 upregulate p53 by activating the NF-κB transcription factor. Recent studies have shown that LMP1 activates the NF-κB transcription factor (23, 34, 44), leading to the expression of several genes, including the long terminal repeat (LTR) of human immunodeficiency virus type 1 (HIV-1) (18); A20, a zinc finger protein which affords protection against the cytotoxic actions of tumor necrosis factor alpha (28); and IκB-related genes (20). A single study reported that EBNA-2 activates the HIV LTR via NF-κB induction (47). It has also been reported that tumor necrosis factor alpha mediates increased p53 levels by activating NF-κB, which then binds to an NF-κB consensus binding site in the p53 promoter (62).

Because of these reports, we evaluated the possibility that EBNA-2 and LMP1 increase p53 levels during the early stages of infection via NF-κB activation. In these studies, 293 primary human embryonal kidney cells were transfected with the EBNA-2, LMP1, or EBNA-2 plus LMP1 expression plasmids by the lipofectamine procedure (Gibco BRL Life Technologies, Inc.). One microgram of each plasmid was used for all combinations, and the vectors (pSV2gpt and pSG5) were used to balance the total amount of DNA. Transfection efficiencies were adjusted to the same values, as determined by the expression of the pCMV-β-gal plasmid.

As shown in Fig. 3A, the 293 transfected cells expressed EBNA-2, LMP1, or both latent genes, as assessed by the Western blotting technique (using EBNA-2 monoclonal antibody PE2 [64], LMP1 monoclonal antibody S12 [33], peroxidase-labeled anti-mouse immunoglobulin G, and the ECL system) 72 h after transfection. Gel shift assays were carried out to determine whether EBNA-2 and LMP1 induced NF-κB-like nuclear proteins. For these studies, nuclear extracts were prepared as described previously (49) and 10 μg of protein per sample was evaluated for the ability to bind a double-stranded end-labeled oligonucleotide duplicating the NF-κB site in the p53 promoter (+49 to +68, 5′-TGGGATTGGGACTTTCC CCT-3′). Competition studies were performed with 50-fold excesses of the unlabeled wt oligonucleotide, an oligonucleotide with mutations in the p53 NF-κB site (5′-TGGGATTAT CACTTTTAAGCT-3′; mutated positions are underlined) and a canonical NF-κB consensus sequence (5′-AGTTGAGGG GACTTTCCCAGGC-3′; Promega). As shown in Fig. 3B, EBNA-2 and, to a greater extent, LMP1 induced the appearance of nuclear proteins which bound to an oligonucleotide containing the NF-κB site in the p53 promoter, and an additive effect was observed in 293 cells expressing both proteins. The binding was specific, as it was fully inhibited by the homologous oligonucleotide and by an oligonucleotide containing the

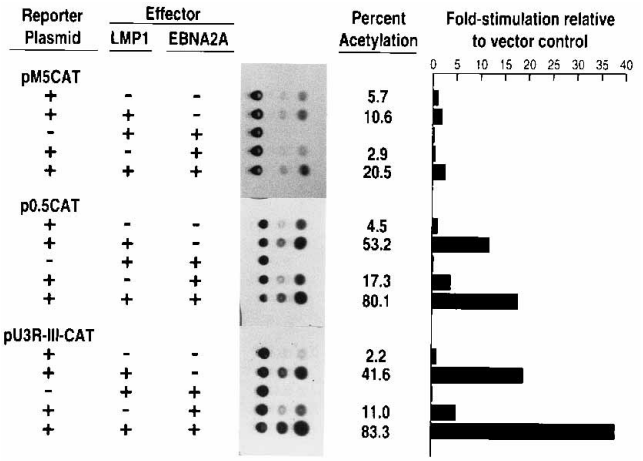

FIG. 4. EBNA-2 and LMP1 independently activate the p53 promoter via NF-κB. CAT assays were performed on 293 cells 72 h after cotransfection with the p53 reporter plasmid p0.5CAT (containing an intact NF-κB site) or pM5CAT (containing a mutant NF-κB site), together with EBNA-2, LMP1, or EBNA-2 plus LMP1 expression plasmids. The HIV LTR construct containing two NF-κB sites, pU3R-III-CAT, was used as a control. Percent acetylation was quantitated by scanning with a PhosphorImager.

NF-κB consensus sequence but not by an oligonucleotide containing a mutated NF-κB sequence, as depicted in Fig. 3B.

In order to determine whether activation of the p53 promoter was NF-κB dependent, 293 cells were cotransfected with the EBNA-2, LMP1, or EBNA-2 plus LMP1 expression plasmids together with one of two chloramphenicol acetyltransferase (CAT) reporter plasmids: p0.5CAT or pM5CAT (62). The p0.5CAT construct contains approximately 500 bp of the wt p53 promoter (−320 to +216), as does the pM5CAT construct; however, in the latter, the sequence GGGACTTTCCC, which contains the NF-κB consensus binding sequence, has been mutated to ATCACTTTAAG. As a positive control, the pU3R-III-CAT construct (43), which contains two NF-κB sites in the HIV LTR, was also cotransfected with EBNA-2, LMP1, or EBNA-2 plus LMP1. Transfections were carried out as described above, and cell extracts were prepared 72 h later. CAT assays were carried out using a CAT enzyme assay system (Promega), as previously described (16). The percent acetylation of thin-layer chromatograms was quantitated by scanning with a PhosphorImager (Molecular Dynamics). These assays showed that the p53CAT promoter construct containing the wt NF-κB binding sequence (p0.5CAT) was activated in cells which had been cotransfected with plasmids containing EBNA-2, LMP1, or both latent genes, as shown in Fig. 4. Transfectants expressing EBNA-2 produced a 3.8-fold increase, those expressing LMP1 exhibited an 11.8-fold increase, and those expressing both latent genes showed a 17.8-fold increase in CAT activity over background levels. However, the p53CAT construct containing the mutated NF-κB site was not significantly activated by either EBV latent gene (3.6-fold maximal increase over background CAT activity), as seen in Fig. 4. EBNA-2, LMP1, and the two together activated the HIV-1 LTR (5-, 18.9-, and 37.9-fold over background, respectively), as previously reported (18, 47). These studies definitively demonstrate that p53 induction by the EBV latent genes is mediated via NF-κB activation.

There are at least three possible explanations for the failure of the increased levels of wt p53 induced by EBV infection to block cell division and/or to induce apoptosis, and they are not mutually exclusive. First, there may be a threshold level of p53 required for these effects which is not reached during EBV infection. Second, downstream events which mediate p53-triggered cell cycle arrest or apoptosis may be blocked by EBV latent proteins or by EBV-induced cellular proteins. Third, and we feel most likely, EBV-induced increases in c-myc (7, 22, 26) or in other cell cycle-regulating proteins, such as cyclin D2, cdc-2, or cyclin E, all of which are increased by EBV infection (22, 26, 41, 50), may block the inhibitory effects of elevated p53 and p21/WAF1 levels and continuously drive the cells through normal cell cycle checkpoints. Similar postulates have been made by others to explain other aspects of EBV immortalization (3, 22, 26). Studies of the mechanisms involved are under way.

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